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Regulatable production of mature insulin from a hepatocyte cell line: insulin production is up-regulated by cAMP and glucocorticoids, and down-regulated by insulin

Danhong Lu, Hideki Hoshino, Toshiyuki Takeuchi*

Department of Molecular Medicine, Institute for Molecular and Cellular Regulation, Gunma University, Showa-machi, Maebashi 371, Japan

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Abstract **We engineered a hepatoma cell line that produces an up-regulation of insulin in response to cAMP, dexamethasone, and retinoic acid, and a down-regulation in response to insulin. We devised a regulatory secretion system by placing proinsulin DNA under the regulatable promoter for phosphoenolpyruvate carboxykinase (PEPCK). To assess the ability to regulate insulin secretion, we used the rat hepatoma cell line, H4IIE. The H4IIE cells secreted immunoreactive insulin (IRI) constantly at a level of 1–3 fmol/10^6 cells/h. IRI increased approximately two-fold upon stimulation with 0.5 mM cAMP and five-fold with the addition of the cAMP-dependent phosphodiesterase inhibitor IBMX, as compared to baseline IRI secretion. IRI increased 18-fold by 1–500 nM dexamethasone together with cAMP and IBMX. Addition of exogenous insulin to the culture medium significantly decreased insulin mRNA expression on Northern blot.**

Key words: Insulin production; PEPCK promoter; H4IIE; Gene therapy; Proinsulin processing

1. Introduction

Two types of cells are available for engineering insulin-producing surrogate cells for diabetes gene therapy: neuroendocrine and non-neuroendocrine cells. Neuroendocrine cells are equipped with a processing mechanism that converts a propeptide into a mature bioactive peptide, and a regulatory secretion system by which a mature peptide is secreted in response to extracellular stimuli. Thus, when neuroendocrine cells, such as the anterior pituitary corticotroph-derived endocrine cells AtT20, are used for diabetes gene therapy, the primary focus has been to engineer an insulin secretion mechanism that responds to a physiological range of glucose stimuli [1–3]. On the other hand, when non-neuroendocrine cells are used as insulin-producing cells, the cells need to be engineered with a processing mechanism and a regulatory secretion system. Several attempts have been made to confer insulin-producing capabilities on non-neuroendocrine cells such as the mouse fibroblast-derived Ltk^- cells [4–6]. The Ltk^- cells transfected with insulin DNA produced proinsulin, but not mature insulin, independent of extracellular stimuli because these cells are not equipped with a processing mechanism or a regulatory secretion system. These issues must be addressed before non-neuroendocrine cells can be used as insulin-producing surrogate cells for diabetes gene therapy.

In pancreatic β cells, the processing of proinsulin into mature insulin consists of two enzymatic reactions: cleavage by the prohormone convertases PC2 and PC3 (also named PC1), and trimming of the carboxyl terminal basic amino acids from the newly exposed B-chain of insulin by carboxypeptidase H (CPH) [7–9]. These two proteolytic reactions are specific to secretory granule-containing neuroendocrine cells [8,10]. Indeed, pioneering work by Moore et al. has demonstrated that human proinsulin can be correctly processed to insulin in the endocrine cell line AtT20, but not in the fibroblast cell line Ltk^- [4]. However, when we replaced the processing sites of proinsulin with those cleavable by the yeast Kex2 family endoprotease furin, the non-neuroendocrine cell lines, including COS-7, HepG2, CHO, and NIH3T3, produced insulin that was the same size as synthetic human insulin [11,12]. Next, the furin-cleaved proinsulin required the removal of basic residues by carboxypeptidases for its maturation. Although non-neuroendocrine cells expressed different quantities of CPH mRNA, these cells contained considerable levels of carboxypeptidase activity. The insulins resulting from these cell lines were eluted as a single peak at the same position on a cation-exchange chromatography column as mature insulin [13]. Thus, non-neuroendocrine cells are able to produce correctly processed insulin if proinsulin is first mutated to possess furin-cleavable processing sites.

For developing an insulin expression system that can be regulated by extracellular stimuli, the most desirable system is to use glucose as a stimulator. Insulin may be used as an alternative regulator for down-regulating its expression. Because non-neuroendocrine cells do not carry secretory granules and secrete proteins and peptides through a constitutive pathway without their retention in the cytoplasm [8], the regulatory step available for the production of insulin is limited to the gene transcription level. The expression of many genes is regulated by glucose [14–16]. For the regulatory expression of these genes, target cells must have both a glucose transporter type 2 and a glucokinase in order to respond to a physiological range of glucose concentrations [3]. To meet these criteria, the primary cultured hepatocytes are the cells of choice for assessing the expression of glucose-regulated genes. Hepatocytes are relatively inefficient, however, for DNA transfer by conventional gene transfer methods [17]. Furthermore, hepatocyte culture cell lines with glucose transporter type 2 and glucokinase are not available at the present time. Thus, we attempted to generate a system in which expression is regulated by insulin, using a hepatoma cell line and the phosphoenolpyruvate carboxykinase (PEPCK) promoter [18–20]. The PEPCK is expressed in hepatocytes and catalyzes oxaloacetate to phosphoenolpyruvate by removing a phosphate from GTP. This enzyme facilitates hepatic glucose production. In diabetic subjects this enzyme is highly active due to the lack of insulin. The PEPCK promoter is known to be

*Corresponding author. Fax: (81) (272) 20-8896.
E-mail: tstake @sb.gunma.ac.jp

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up-regulated by cAMP, glucocorticoids, and retinoic acid (RA), and is down-regulated by insulin [20–22]. For assessing the PEPCK promoter activity, several hepatocyte culture cell lines are available, including H4EII and FTO2B. Thus, we examined the insulin production using a PEPCK promoter plus an insulin DNA construct in H4EII cells.

Experimental gene therapy for diabetes was first attempted by Selden et al. by expressing a metallothionein promoter plus a human insulin DNA construct in mice [5]. This expression vector was introduced into Ltk^- cells together with a thymidine kinase gene. The resultant Ltk^+ cells that produced proinsulin were injected intraperitoneally into diabetic C3H mice, which were immunosuppressed with the use of rabbit anti-mouse thymocyte serum and dexamethasone. The mice exhibited normoglycemia a week after the implantation of proinsulin-producing Ltk^+ cells, but died from the growth of Ltk^+ cells or from hypoglycemia [6]. Other types of experimental gene therapy trials have been attempted using liver cells with a liposome-Sendai virus gene transfer system or a retroviral system [23,24]. None of the gene therapy trials, however, utilized efficient, regulatable insulin expression systems.

In the present study we demonstrate that insulin production in H4EII cells under the PEPCK promoter was up-regulated by cAMP, IBMX, and dexamethasone, and down-regulated by insulin. When we expressed the proinsulin with furin-cleavable processing sites in this cell line, the cells produced mature insulin.

2. Materials and methods

2.1. Construction of a regulatable insulin expression vector

A regulatable insulin expression vector was made by modifying a previously reported insulin expression vector [12]. The previous vector contains a rat insulin gene I with or without furin-cleavable sites [12]. We inserted a thymidine kinase (TK) promoter (−105 to +51) as a basal promoter, and placed a PEPCK promoter (−598 to −31) before the TK promoter. The PEPCK promoter was amplified by PCR using the PEPCK promoter-containing plasmid pPI.9 CAT (a kind gift from Dr. Daryl Granner) [25]. We constructed six different expression vectors with multiple copies of PEPCK promoters.

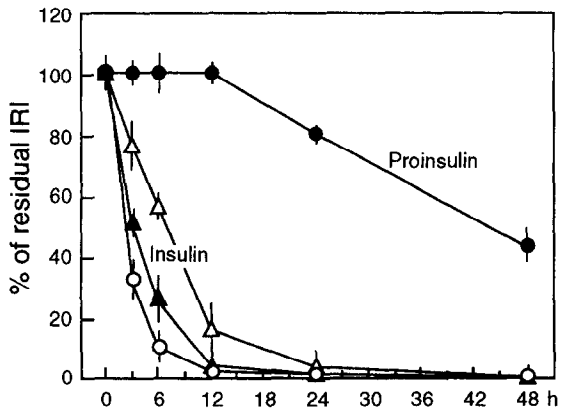

Fig. 1. Disappearance of IRI from the culture medium of H4IIE cells. The intact H4IIE cells were cultured in the presence of human insulin or human proinsulin. Proinsulin was added to the culture medium at a final concentration of 5 nM at the 0 time point. Insulin was added at final concentrations of 0.2, 1, and 5 nM. At each indicated time, samples were collected from the medium for IRI measurement. Percent of residual IRI in the medium is expressed relative to initial IRI (0 time point). Proinsulin 5 nM, closed circles; insulin 5 nM, open triangles; 1 nM, closed triangles; 0.2 nM, open circles. Bars indicate standard deviation.

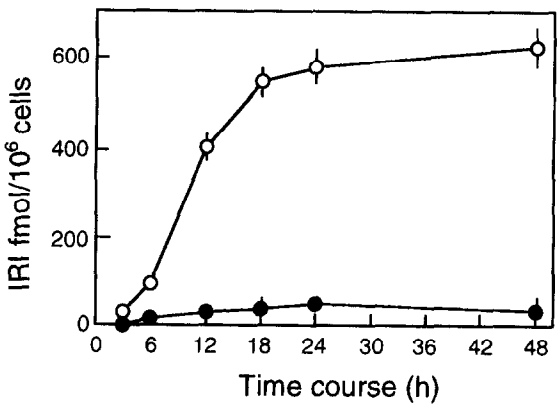

Fig. 2. Time course of IRI production from the cells transformed with the P2/TK/PI DNA. IRI production was chased for 48 h. To examine the regulated IRI production one group of cells were cultured in the presence of 0.5 mM cAMP+0.5 mM IBMX+500 nM dexamethasone (open circles), the other cultured in the absence of the stimulants (closed circles). Bars indicate standard deviation.

2.2. Cell culture and DNA transfection

The rat hepatoma cell line H4IIE (ATCC CRL 1548) was used in this study. This cell line is frequently used for PEPCK promoter analysis with a variety of regulators [20–22]. The H4IIE cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) with 6% fetal bovine serum (FBS; Gibco, Grand Island, NY) at 37°C in 5% CO_2. Glucose concentration in the medium was 25 mM. Cells were transformed using a liposome method with DOTAP *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium-methyl sulfate (Boehringer Mannheim, Indianapolis, IN). Because the vectors contained a neomycin-resistant gene, the native or mutant proinsulin-expressing cells were selected by maintaining the culture in a medium containing the neomycin analogue G418 (Gibco; 0.5 mg/ml).

2.3. Radioimmunoassay

Immunoreactive insulin (IRI) in the culture medium was detected using an insulin immunoassay kit (Amersham Japan, Tokyo, Japan). The antibody in this kit recognizes both proinsulin and mature insulin on an equimolar basis.

2.4. Regulation of IRI production

To observe the regulatable production of IRI, we added the following chemicals to the culture medium: the cAMP analogue N^6,2,0-dibutyryl cyclic adenosine monophosphate (dbcAMP), the cAMP-dependent phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, retinoic acid, human insulin. All chemicals were purchased from Sigma, St. Louis, MO. The H4IIE cells were incubated with each chemical for 18 h, unless otherwise stated.

2.5. Northern blot

The isolation of the total cellular RNA from each cell line was performed as described previously [12]. The total RNA was electrophoresed on a 1.2% agarose gel, and transferred to a nylon membrane (Amersham). Hybridization was performed with a probe of the rat insulin gene I DNA fragment (350 bp) labeled with [α-^{32}P]deoxy-CTP by the random priming procedure. The membrane was exposed to an X-ray film (Eastman Kodak, Rochester, NY) with an intensifying screen at −80°C.

2.6. Gel filtration

Gel filtration chromatography was carried out on a 1.0×120 cm column (Bio-Rad) containing Sephadex G-50 superfine gel (Pharmacia LKB, Piscataway, NJ), equilibrated with 100 mM acetic acid, to analyze the apparent size of insulin secreted into the culture medium. Eluted samples were assayed for IRI with RIA.

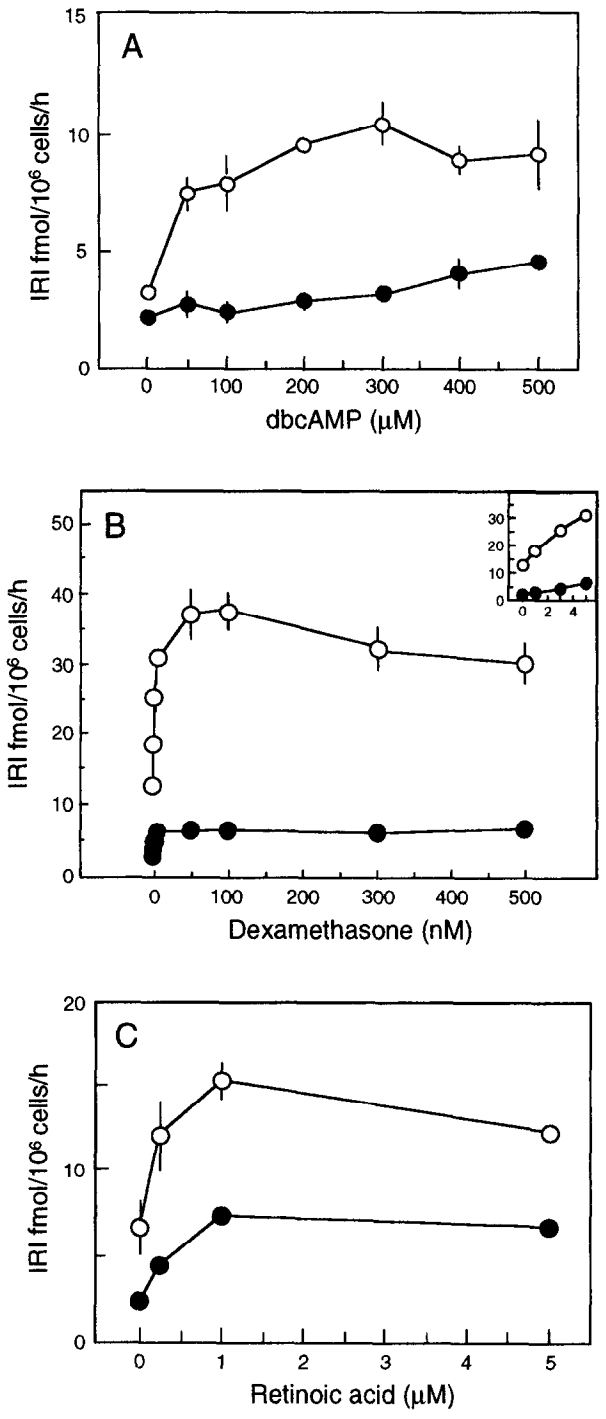

Fig. 3. Regulated production of IRI with cAMP, dexamethasone, and retinoic acid. The H4IIE cells transformed with the P2/TK/PI DNA were cultured with or without the stimulants. After 18 h incubation, samples were collected from the culture medium for IRI measurement. A: The H4IIE cells were stimulated with cAMP alone or cAMP+0.5 mM IBMX. cAMP was added to the medium at final concentrations of 50–500 mM. Closed circles: culture with cAMP alone; open circles: culture with cAMP+0.5 mM IBMX. Bars indicate standard deviation. B: The cells were stimulated with dexamethasone alone or dexamethasone+0.3 mM cAMP+0.5 mM IBMX. Dexamethasone was added to the medium at final concentrations of 1–500 nM. Closed circles: culture with dexamethasone alone; open circles: culture with dexamethasone+0.3 mM cAMP+0.5 mM IBMX. Bars indicate standard deviation. Inset: blowup of IRI production curves between 0 and 5 nM dexamethasone. C: The cells were stimulated with retinoic acid alone or retinoic acid+0.3 mM cAMP+0.5 mM IBMX. Retinoic acid was added to the medium at final concentrations of 0.2–5.0 mM. Closed circle, cultus:e with retinoic acid alone; open circles: culture with retinoic acid+0.5 mM cAMP+0.5 mM IBMX. Bars indicate standard deviation.
←

3. Results

3.1. Regulatable production of IRI

In previous studies we noted that IRI from the mutant insulin-expressing cells was much lower than that from the native proinsulin-expressing cells [11,12]. It may be that the culture cells adsorb mature insulin by insulin receptor-mediated endocytosis, thereby secreting less insulin into the culture medium. We tested this possibility by adding proinsulin and insulin into the H4IIE culture medium. Mature insulin disappeared quickly from the culture medium, whereas proinsulin decreased much more slowly than mature insulin until 48 h after insulin or proinsulin was added to the H4IIE culture medium (Fig. 1). Thus, for the assessment of regulated IRI production, we used the cells transformed with the proinsulin expression vector first, then evaluated the autoregulatory feature of insulin production using the native type insulin-producing cells transformed with the insulin expression vector.

Among the six cell lines we established, the greatest increase of IRI was observed in the cell line expressing a vector with two PEPCK promoters+TK promoter+proinsulin DNA (P2/TK/PI) when stimulated with 0.5 mM dbcAMP+0.5 mM IBMX+500 nM dexamethasone. Thus, we used this cell line for the assessment of regulatable IRI production. Firstly, we examined IRI production over time. A low level (1–3 fmol/10^6 cells/h) of IRI was produced over time by the proinsulin-producing cells. Upon stimulation with dbcAMP+IBMX+dexamethasone, IRI production increased linearly until 18 h after the stimulation, then plateaued gradually towards the 48-h point (Fig. 2). Thus, we evaluated IRI production using the medium after 18 h in culture.

IRI production gradually increased 2-fold with increasing amounts of dbcAMP (up to 500 μM; Fig. 3A). When the cAMP-dependent phosphodiesterase inhibitor, IBMX, was added to dbcAMP, the increase in IRI was more marked. IBMX alone (up to 1.5 mM) did not increase IRI production; thus, 0.5 mM IBMX was used in the present study. In the presence of IBMX, 50 μM dbcAMP induced approximately a 2.5-fold increase in IRI and 300 μM dbcAMP induced a greater than 3-fold increase as compared to treatment with 300 mM dbcAMP alone (Fig. 3A). Although the addition of 0.5 mM IBMX induced a similar increase between 50 and 500 μM dbcAMP, 300 μM dbcAMP+0.5 mM IBMX induced a relatively high increase. Thus, we used the dose of 300 μM dbcAMP in the treatment study.

The addition of dexamethasone (1–5 nM) induced approximately a 3-fold increase. Increasing the dose of dexamethasone (up to 500 nM) did not induce further increases (Fig. 3B). The addition of dexamethasone with 300 μM dbcAMP and 0.5 mM IBMX produced an enhanced increase in IRI production, which reached approximately 4-fold over the level induced by 5 nM dexamethasone alone, and approximately 18-fold over the IRI level produced with no stimulants (Fig.

3B). The greatest increase of IRI was obtained at 50 and 100 nM dexamethasone with 300 μM dbcAMP+0.5 mM IBMX. Thus, we used 100 nM dexamethasone for the combined stimulants. Retinoic acid (up to 1.0 μM) was also effective in increasing IRI production (Fig. 3C). The combination of dbcAMP and IBMX was effective in enhancing the efficacy of retinoic acid. The combination increased IRI production approximately 7-fold over the basal production without stimulation.

3.2. Suppressive effect of insulin on insulin mRNA expression

It is not possible to evaluate the inhibitory effects of insulin on IRI production unless RIA of rat proinsulin C peptide is available. Thus, we measured insulin mRNA expression in the H4IIE cells with the increase of exogenous insulin (0, 0.1, 1.0, 10 nM) in the culture medium. The expression of insulin mRNA decreased with increased levels of an exogenous insulin dose (Fig. 4A). Even with 0.1 nM insulin, the message was so inhibited as to be barely visible on the blot. However, the inhibition by exogenous insulin was less effective when the stimulants were added simultaneously with insulin. The insulin message stayed unchanged with 0, 0.1, and 1.0 nM exogenous insulin, but the message decreased distinctly with 10 nM of insulin in the culture medium. When the cells were treated with insulin for 8 h, then with the stimulants but without insulin for another 18 h, the inhibitory effect of insulin continued extensively (Fig. 4, lane −8 h). Thus, overdose of insulin is effective in suppressing the insulin mRNA expression.

3.3. Regulated insulin production from the native or mutant proinsulin-expressing cell line

Since we found that the proinsulin expression vector carrying the P2/TK/PI DNA regulated the expression in the H4IIE cells, we replaced the mutated proinsulin DNA encoding the furin-cleavable sites for the native proinsulin DNA in the expression vector (P2/TK/I). As expected, the cells expressing this vector produced a more mature type of insulin (Fig. 5B). Approximately 60% of the IRI was eluted at the native insulin position on gel filtration chromatography, suggesting that the H4IIE cells may contain a high level of furin [12]. In contrast, the P2/TK/PI-expressing cells produced IRI, more than 90% of which corresponded to the proinsulin position on the gel (Fig. 5A).

IRI production from the P2/TK/I-expressing cells was also regulated in a similar manner to that from the proinsulin-expressing cells by dbcAMP and dexamethasone, as shown in the left panel of Fig. 6. However, the increase of IRI by the stimulants was less than that observed in P2/TK/PI-expressing cells. For example, IRI was increased only about 6-fold more by the stimulants dbcAMP+IBMX+dexamethasone than that from the cells without stimulation (ABD in Fig. 6A). In contrast, the increase of proinsulin was 15-fold more by the same stimulants than that from the non-stimulated cells (ABD in Fig. 6B). Moreover, the IRI produced from the P2/TK/I-expressing cells was about a third to a fifth of that of the P2/TK/PI-transformed cells producing native proinsulin probably due to endocytosis depicted in Fig. 1.

4. Discussion

To engineer non-neuroendocrine cells for use as insulin-producing surrogate cells, the cells must be able to: (1) process proinsulin, (2) regulate insulin secretion and (3) efficiently accept transfer of the insulin expression vector. Proinsulin was processed using ubiquitously distributed endoprotease furin. For the processing of proinsulin by furin, the dibasic residues between the B chain and C peptide and between C peptide and the A chain were mutated to tetrabasic residues to allow for furin cleavage [11,12,26,27].

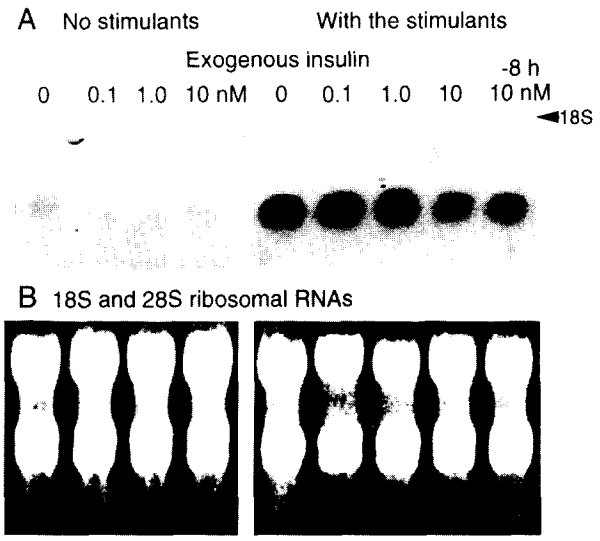

Fig. 4. Northern blot analysis of the insulin mRNA expression with exogenous insulin in the culture medium. The H4IIE cells transformed with P2/TK/PI DNA were cultured with exogenous insulin for 18 h at final concentrations of 0, 0.1, 1.0, 10 nM in the presence or absence of the stimulants; 0.3 mM cAMP+0.5 mM IBMX+100 nM dexamethasone. Insulin was added to the culture medium together with the stimulants. Total RNAs of 20 μg from each cell group were used in this experiment. A: The left panel illustrates the blotting from the cell culture in the absence of the stimulants, the right panel that in the presence of the stimulants. 18S represents the corresponding size of ribosomal RNA. The far right lane marked −8 h indicates that the cells were cultured in the presence of 10 nM insulin for 8 h, then for 18 h with the stimulants but without insulin. B: 28S and 18S ribosomal RNA bands stained with ethidium bromide.

The secretion of insulin from non-endocrine cells should be increased by a high concentration of glucose and decreased by a low concentration of glucose. Because the regulating step for insulin secretion is limited to the level of gene transcription in non-neuroendocrine cells, we examined several candidate promoters with a glucose-responsive element for regulating insulin production. For example, we used a promoter for rat L-type pyruvate kinase because it contains two glucose-responsive elements [28]. But an attempt to regulate insulin expression by this promoter was not successful because glucose activation of the glucose-responsive element was weak, even if multiple elements were placed ahead of the basic promoter. Thus, we used a PEPCK promoter because this promoter is known to be activated by cAMP, glucocorticoids, and retinoic acids, and suppressed by insulin [14].

As the first step, we expressed native proinsulin to reduce insulin receptor-mediated endocytosis because proinsulin has a much lower affinity for the receptors than does mature insulin, and does not suppress the PEPCK promoter as much as mature insulin [29]. DbcAMP, dexamethasone, or their combination stimulated the increase of IRI production only 2–3-

fold. The addition of IBMX to dbcAMP, or to dbcAMP+dexamethasone, however, induced a significant increase in IRI production (Fig. 3). In previous studies using H4IIE cells, PEPCK promoter activity increased 8–16-fold over basal activity when stimulated by the addition of 0.1 mM 8-(4-chlorophenylthio)-cAMP+0.5 mM dexamethasone, and reduced 80–90% from the maximal activity by the addition of 5 nM insulin in several assay systems, including dot blot hybridization and CAT activity assay [20,29]. Thus, our data using insulin as a reporter are consistent with previous reports. Although insulin is known to suppress PEPCK promoter activity [20,21], insulin-induced inhibition of insulin is difficult to measure in our engineered cells. Thus, Northern blot analysis was used to assess decreased expression of insulin mRNA by exogenous insulin in culture medium (Fig. 6). We observed a decreased expression of insulin mRNA with a high dose of exogenous insulin (10 nM).

Another obstacle to overcome is the efficient gene transfer in hepatocytes [17]. For this purpose a retroviral or adenoviral transfer method, or a liposome-mediated transfer method may be suitable [23,24,30]. We are currently in the process of examining these methods using diabetic animals. The insulin expression vector we devised may be useful for an accessory insulin-producing system in gene therapy or in hybrid-type artificial islets for the treatment of diabetes mellitus.

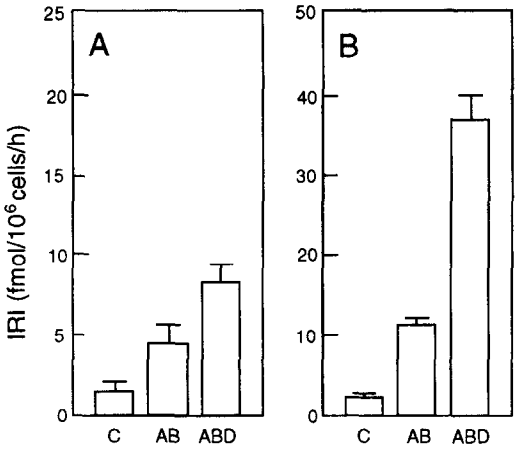

Fig. 6. Regulatable IRI production from the insulin-producing cells and proinsulin-producing cells. The cell line transformed with the P2/TK/I DNA was used as insulin-producing cells. IRI production from the insulin-producing cells was compared with that from the proinsulin-producing cells transformed with the P2/TK/PI DNA. Panel A illustrates the IRI production from the insulin-producing cells, panel B illustrates IRI production from the proinsulin-producing cells. A, 0.3 mM cAMP; B, 0.5 mM IBMX; D, 500 nM dexamethasone.

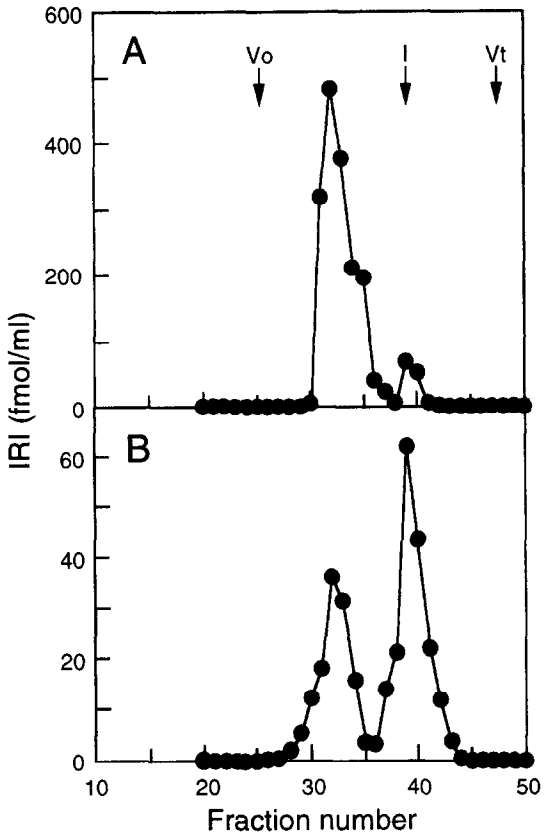

Fig. 5. Gel filtration profiles of IRI in the culture medium of the P2/TK/PI- or P2/TK/I-expressing cells. The concentrated media were applied to Sephadex G-50 superfine gel columns (1.0×120 cm) equilibrated with 0.1 M acetic acid. Fractions of 1.5 ml were collected and measured for IRI by RIA. A: IRI from the P2/TK/PI-expressing cells; B: IRI from the P2/TK/I-expressing cells. Molecular size was calibrated with blue dextran (Vo), potassium ferricyanide (Vt), and synthetic human insulin (I). Similar elution profiles were obtained in at least three other gel filtrations for each experiment.

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